

Using a Portable Real-Time PCR Assay To Detect *Salmonella* in Raw Milk[†]

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ABSTRACT

The purpose of this study was to determine the efficacy of a portable real-time PCR system in detecting *Salmonella* spp. in raw milk. The 200 bulk milk samples chosen for this study constituted a subset of the samples for a larger study; this subset contained 24 samples that were culture positive for *Salmonella* and 176 that were culture negative. Milk was both plated directly on selective agar and plated after enrichment in selective media. Presumptive *Salmonella* colonies were isolated by direct culturing of five samples, while *Salmonella* was isolated from the remaining 19 positive samples only after enrichment. Presumptive *Salmonella* isolates were serotyped, and isolates from 22 samples were confirmed to be *Salmonella* isolates. PCR assays of culture-positive milk prior to enrichment yielded no evidence of *Salmonella*. DNA extracts of bacterial pellets from the enriched samples were analyzed for *Salmonella* by real-time PCR with the Ruggedized Advanced Pathogen Identification Device (RAPID). Fifty-four samples from the enrichment pellets tested positive for *Salmonella* by real-time PCR. Two samples that tested positive for *Salmonella* by culture and serotyping tested *Salmonella* negative by real-time PCR. Serotyping identified isolates from these samples as *Salmonella* Montevideo. All DNA extracts of *Salmonella* Montevideo isolates tested positive for *Salmonella* by real-time PCR. Thirty-three samples tested negative by culture and positive by real-time PCR. These results indicate that the portable real-time PCR system appears to be a useful tool for detecting *Salmonella* in raw milk. Additionally, the combination of enrichment and real-time PCR techniques used in this study can yield results in 24 h, compared with the 48 to 72 h required for traditional culture.

Salmonellosis is a common illness affecting more than one million people annually in the United States (11), and financial costs attributable to this disease are substantial. The majority of cases are due to the consumption of contaminated animal products such as eggs, poultry, raw meats, raw milk, and other dairy products that have not been pasteurized or have been handled inappropriately (3, 4). Milk and milk products have been identified as the vehicle for transmission in approximately 5% of salmonellosis cases, although the sources of infection remain unidentified in most cases (4).

Salmonellosis is commonly diagnosed in dairy cows and calves, and the presence of *Salmonella* on dairy farms has been well documented (8, 10, 19). Salmonellosis in cattle can result in gastritis, abortion, decreased milk production, or even death, but fecal shedding of *Salmonella* by asymptomatic animals has also been observed (7). Although there is evidence that *Salmonella* is shed from the mammary gland (13, 16), fecal contamination is also likely a major source of contamination of raw milk.

Studies of the prevalence of *Salmonella* in farm bulk tank milk have yielded various results: surveys conducted in Tennessee, Tennessee and Virginia, South Dakota and

Minnesota, and Ontario showed, respectively, 2.24, 8.9, 6.1, and 0.17% of bulk tank milk to be contaminated with *Salmonella* (9, 12, 14, 17). Although the majority of milk consumed is pasteurized, many farm families drink raw milk (9), and there is a small but growing public interest in the consumption of raw milk (1). Soft Mexican-style cheeses are often made with unpasteurized milk, and several reported outbreaks of salmonellosis have resulted from the consumption of such cheeses (5, 18).

Currently, the testing of farm bulk milk for zoonotic pathogens such as *Salmonella* is not routine. Raw milk is typically monitored for total aerobic counts or other bacterial groups (i.e., total coliforms) as bacterial load indicators (20); specific cultures are usually used only when a potential problem arises. As concern about foodborne pathogens increases, the regular monitoring of bulk tank milk for individual organisms may become more desirable.

Traditional culture methods for *Salmonella* detection are laboratory-based and are generally time-consuming and labor-intensive. Recent advancements in PCR technology may allow more rapid, and perhaps even on-farm, detection of foodborne pathogens. The purpose of this study was to determine the efficacy of a portable real-time PCR system for the detection of *Salmonella* in raw milk.

MATERIALS AND METHODS

Samples. The 200 milk samples used in this study constituted a subset of samples from the National Animal Health Monitoring System Dairy 2002 survey. The selection of samples was

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not random in that 24 samples that had tested *Salmonella* positive by culture and 176 samples that had tested negative by culture were chosen. Bulk tank milk (50 ml) was collected from dairy farms in 21 states and shipped overnight with cold packs to the USDA Agricultural Research Service laboratory in Beltsville, Md. Samples were collected from March to June of 2002. In some cases, the samples were frozen prior to shipping. Upon arrival at the laboratory, milk samples were immediately partitioned for various analyses.

Bacteriological methods. Milk samples (250 μ l each) were plated in triplicate directly onto XLT4 agar (XLT4 agar base with XLT4 supplement; Difco Laboratories, Detroit, Mich.) with an Autoplate 4000 (Spiral Biotech, Norwood, Mass.). Plates were incubated at 37°C and scored for presumptive *Salmonella* colonies (black colonies) after 24 and 48 h. For the enrichment of *Salmonella*, 5 to 10 ml of milk was added to 95 ml of tetrathionate broth. The variation in volume was due to variation in amounts of available samples: the initial volumes of some of the samples were <50 ml. Enrichment bottles were incubated at 37°C for 24 h, and then 10- μ l portions of broth were streaked onto XLT4 agar. Plates were incubated and scored as described above.

Isolated presumptive *Salmonella* colonies were patched from XLT4 plates onto XLT4 agar, brilliant green agar, and L-agar (Lennox Broth base with 1.5% agar; Gibco Laboratories, Long Island, NY). Colonies that exhibited the *Salmonella* phenotypes (black on XLT4 agar and pink on brilliant green agar) were preserved for future analysis. Colony biomass was transferred from the L-agar plates to a vial containing a 1:1 mixture of Lennox Broth and the 2X Freezing Medium for Cells of Schleif and Wensink (15), and the isolates were stored at -80°C. L-agar slants were inoculated and, after incubation at 37°C for 24 h, sent to the National Veterinary Services Laboratories in Ames, Iowa, for serotyping.

Enriched samples (1.5 ml each) were centrifuged at 16,000 \times g in microcentrifuge tubes, the supernatants were discarded, and the pellets were stored at -20°C. DNA was extracted from the bacterial pellets with a commercially prepared extraction preparation (InstaGene Matrix, Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's directions. The DNA preparations (200 μ l) were stored at -20°C and were analyzed for the presence or absence of *Salmonella* via real-time PCR at a later date.

Unenriched milk samples ($n = 45$) were also analyzed directly for the presence or absence of *Salmonella* by real-time PCR. Milk (1.0 ml) was centrifuged at 16,000 \times g in a microcentrifuge tube, and the supernatants were discarded. Pellets were reconstituted in 100 μ l of sterile water. DNA was extracted from the entire 100- μ l sample with the IT 1-2-3 RAPID Purification Kit (Idaho Technology Inc., Salt Lake City, Utah) according to the manufacturer's directions. The final elution volume was 100 μ l, and 5 μ l was used in the real-time PCR reaction.

Real-time PCR was carried out with the Ruggedized Advanced Pathogen Identification Device (RAPID; Idaho Technology), a portable, self-contained instrument designed for in-field analysis. Access to electricity (120 V AC) is required to operate the RAPID. The detection sensitivity of the real-time PCR analysis with the RAPID was determined by spiking raw milk samples (1 ml each) with various amounts of *Salmonella* Typhimurium as suspensions in phosphate-buffered saline prepared from cultures grown overnight in Lennox broth. The samples were processed as described above for the unenriched milk samples.

Premixed freeze-dried reagents specific for *Salmonella* (RAPID System-*Salmonella* Detection Kit, Idaho Technology) were used according to the manufacturer's directions. The RAPID

TABLE 1. Results of *Salmonella* analysis of bulk milk by traditional culture techniques and real-time PCR ($n = 200$)

Detection results	No. of samples
Culture	
Direct-plating positive, enrichment negative	3
Direct-plating positive, enrichment positive	2
Direct-plating negative, enrichment positive	19
Total culture-positive milk samples	24
Confirmed <i>Salmonella</i> positive via serotyping	22
Enrichment culture and real-time PCR	
Total PCR positive	54
Culture positive, serotype positive, PCR positive	20
Culture negative, PCR positive	33
Culture positive, serotype negative, PCR positive	1
Culture positive, serotype positive, PCR negative	2
Culture positive, serotype negative, PCR negative	1

assay targets the *spaQ* gene in salmonellae, a part of the chromosomal *inv/spa* complex that is partly responsible for the virulence of the organism. Preincubation was carried out at 94°C for 60 s and was followed by 45 PCR cycles of 95°C for 0 s followed by 60°C for 20 s, with a temperature transition rate of 20°C per s. Other variable parameters included Channel 2, Gain 8, and Mode 1.

The RAPID in conjunction with the Idaho Technology *Salmonella* Detection Kit has the capability to run melting-point curves for the PCR reaction products. Melting-point curves were run for all samples identified as being *Salmonella* positive by the RAPID software. The initial temperature was 94°C for 1 min, and then the temperature was ramped from 50 to 94°C at a rate of 0.2°C per s. A combination of the melting-point curve analysis results and the PCR results was used to manually determine whether a sample was *Salmonella* positive or *Salmonella* negative.

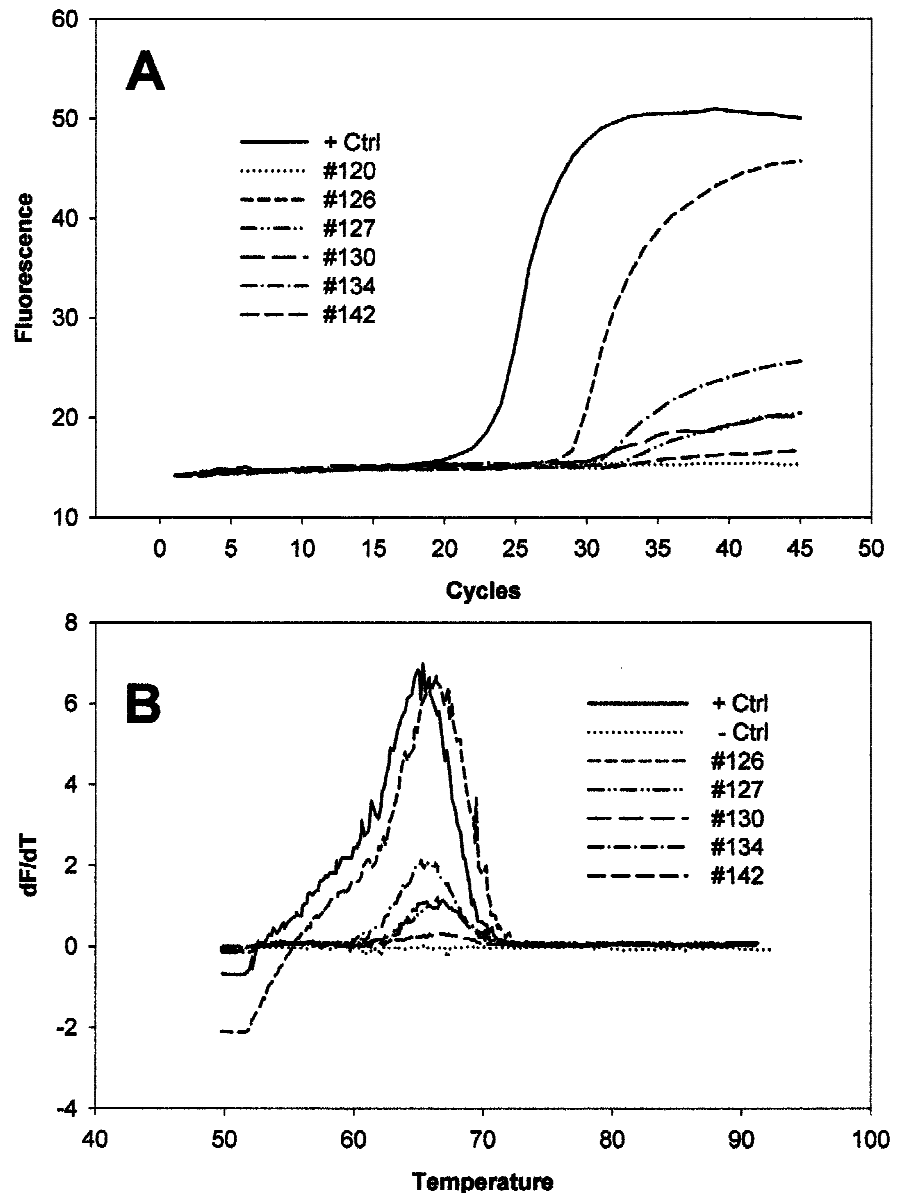
To determine whether the real-time PCR method was differentiating between *Salmonella* Montevideo isolates, at least two isolates from each of the seven samples from which *Salmonella* Montevideo was isolated were analyzed via real-time PCR. Isolates were grown in L-broth, and 1.5-ml samples were centrifuged at 16,000 \times g for 3 min. The supernatants were discarded, and the pellets were extracted and tested for the presence or absence of *Salmonella* spp. as described above for the enrichment pellets.

RESULTS

With traditional culturing techniques, presumptive *Salmonella* isolates were obtained directly from five of the raw bulk milk samples without enrichment (Table 1). Serological analysis confirmed that the isolates from four of these samples were *Salmonella* isolates, while the fifth H₂S-forming organism was not *Salmonella*. Isolates from two of the samples were determined to be *Salmonella* Montevideo, another was *Salmonella* Dublin, and the last was *Salmonella* 9,12:nonmotile.

When an enrichment step was carried out prior to plating, 19 more samples were determined to be presumptively culture positive for *Salmonella* (Table 1). Isolates from 18 of these presumptive positive milk samples were confirmed to be *Salmonella* via serology. The most common serotypes were *Salmonella* Montevideo and *Salmonella* Newport, which were identified in five and four samples, respectively.

FIGURE 1. (A) Plot of fluorescence intensity during real-time PCR run for DNA extracted from enrichment pellets of selected raw bulk tank milk samples. Samples were selected to demonstrate the range of results obtained. (B) Plot of the slope of the fluorescence intensity curve (dF/dT) versus temperature during a melting curve run with the RAPID for the same sample set represented in panel A. Sample 120 was replaced with the negative control because melting-point curves were constructed only for positive samples.



Salmonella Muenster, *Salmonella* Meleagridis, *Salmonella* Cerro, and *Salmonella* 44:Z36 (Z38) were identified in two milk samples each, while *Salmonella* Dublin and *Salmonella* Anatum were identified in one milk sample each. Interestingly, *Salmonella* was not isolated from the tetrathionate enrichments of three samples that tested positive by direct culture.

When DNA extracts of pellets from tetrathionate broth-enriched milk samples were analyzed by real-time PCR, 54 milk samples tested positive for *Salmonella*. Twenty of the 22 raw milk samples that had been shown to contain *Salmonella* by traditional culture techniques were also identified as being *Salmonella* positive by real-time PCR. *Salmonella* was not detected by real-time PCR for two samples that had tested positive for *Salmonella* by culture and serotyping. Isolates from both of these samples were *Salmonella* Montevideo isolates. In one case, the isolated *Salmonella* strain was obtained by direct plating of the milk and no salmonellae were isolated from the enriched sample. In the other case, the *Salmonella* strain was

isolated only after enrichment of the milk. In both of these cases, only one colony was detected, indicating that levels of viable *Salmonella* were very low. In all cases, the *Salmonella* Montevideo isolates were identified as *Salmonella* when DNA extracts of these cultures were analyzed by real-time PCR (data not shown).

Thirty-three of the 176 samples testing negative for *Salmonella* by enrichment and plating subsequently tested positive for *Salmonella* by real-time PCR. Preserved enrichments of these culture-negative, PCR-positive samples were removed from the freezer, and 0.1-ml portions were plated on XLT4 agar. A presumptive *Salmonella* isolate was obtained from only one of these samples, and this isolate was confirmed to be *Salmonella* Cerro. In the remaining 32 samples, either no growth or considerable growth of non-salmonella bacteria was observed.

Figure 1 shows representative real-time PCR runs for extracts from tetrathionate enrichments of bulk tank milk samples. The point at which the curve rises above the baseline is directly related to the amount of *Salmonella* DNA

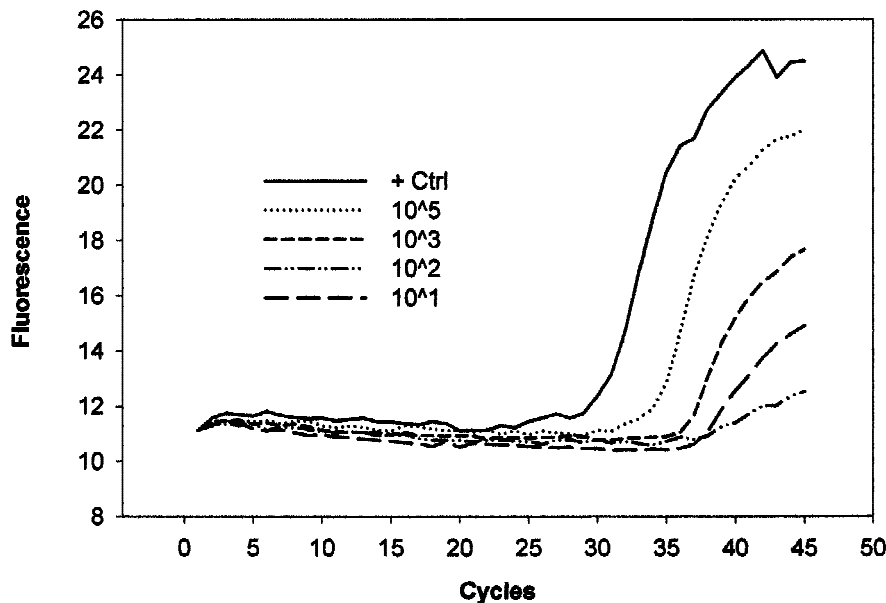


FIGURE 2. Plot of fluorescence intensity during a real-time PCR run for DNA extracted from *Salmonella Typhimurium*-spiked raw milk.

present, while the slope of the curve is related to the efficiency of the PCR reaction. The RAPID software selects a series of points as representative of the baseline and extrapolates this series to the end of the run. The software then compares the magnitude of the fluorescence at the end of the reaction with the magnitude of the baseline predicted by extrapolation to determine whether a reaction was positive or negative. In most cases, when the RAPID software identified a sample as being *Salmonella* positive, manual examination of the real-time PCR plots showed that this result was reasonable (Fig. 1A). However, occasionally, samples that were clearly *Salmonella* negative were identified as positive on the basis of the plots, and few samples judged manually to be *Salmonella* positive were identified as negative. In these cases, an examination of the melting-point curves for the final PCR products (Fig. 1B) was helpful in determining whether the reactions were positive or negative.

When raw bulk tank milk was spiked with 10^1 to 10^5 *Salmonella Typhimurium* cells per ml, real-time PCR analysis with the RAPID detected *Salmonella* in all cases (Fig. 2). Direct real-time PCR analysis of DNA extracted from 45 raw bulk tank milk samples never detected *Salmonella*, even when presumptive positive isolates were obtained by traditional culture methods. As a result, the remaining 155 samples were not directly run in order to avoid wasting costly reagents.

DISCUSSION

Presumptive *Salmonella* strains were isolated from only five of the bulk milk samples in this study by direct plating, indicating that *Salmonella* concentrations in the contaminated samples were relatively low (≤ 400 cells per 100 ml). Selective enrichment was required for the detection of *Salmonella* in 19 of the 24 *Salmonella*-positive samples. Since the enrichment volumes varied from 5 to 10 ml, the lower limit of detection was 10 to 20 cells per 100 ml of milk with this procedure. With the required enrichment step, the detection of presumptive *Salmonella* isolates took

at least 48 h. Following isolation, additional time for biochemical or serological confirmation was necessary, adding another 24 to 48 h.

Milk is a perishable product that cannot be held indefinitely. Timing is critical for the processing of a quality product, and any effort to carry out routine monitoring must take this into consideration. The development of PCR protocols for detecting organism-specific nucleic acid sequences has, in many cases, dramatically decreased the time necessary for the detection of bacteria. These methods are not necessarily more sensitive than culture methods and may still require enrichment; however, postenrichment identification can be completed in a few hours, as opposed to 24 to 48 h.

In recent years, efforts to develop portable detection instrumentation have increased. The RAPID was developed as a compact mobile instrument for running real-time PCR reactions. These reactions take place in capillary reaction vessels and involve target-specific freeze-dried reagents. The mobility of the RAPID allows its use in response to an emergent short-term need for the analysis of a particular organism; such a situation occurred recently when the RAPID was used in a mobile laboratory setting during the period when the Washington, D.C., region was threatened with anthrax (6). Such equipment may be useful for on-farm sampling when intensive sampling is required to determine the cause or extent of an outbreak. With the currently available systems, enrichments and DNA extractions are required, necessitating technical skill and specialized equipment.

The results of the spiking experiment indicate that the real-time PCR method used in this study was capable of detecting as few as 10^1 *Salmonella* cells per ml of sample when milk was sampled directly. Because PCR results for DNA extracted directly from 45 milk samples were negative even though 2 of these samples were culture positive, either *Salmonella* was present at a level below the detection limit (10^1 /ml) or PCR-inhibitory compounds were being extracted along with the DNA. Because bulk tank milk was

used in the spiking experiments, it is unlikely that inhibitory compounds were responsible for the failure to detect *Salmonella* in these two samples. Neither of these positive samples yielded *Salmonella* upon direct plating, indicating that the levels of *Salmonella* were very low. Thus, the failure to detect *Salmonella* was most likely due to the low levels of the organism in the milk. However, differences in milk samples, such as those involving the load of total bacteria or somatic cells, cannot be ruled out as a source of variation in real-time PCR.

Results of real-time PCR runs for DNA extracts of bacterial pellets from the enriched milk samples indicated that 33 culture-negative samples were positive for *Salmonella*, and it appeared that real-time PCR was more sensitive than the culture method used in this experiment. However, attempts to go back and isolate *Salmonella* from 33 samples that tested positive for *Salmonella* by real-time PCR and negative by culture were successful in just one instance. The detection of presumptive salmonellae on XLT4 medium depends on H₂S production, so it is possible that non-H₂S-producing salmonellae were present. Alternatively, the PCR-positive, culture-negative samples may have contained dead or nonculturable salmonellae at very high levels. Perhaps enrichment in a nutrient-rich recovery broth prior to XLT4 enrichment would have yielded more *Salmonella*-positive samples.

The detection of nonviable cells is a well-recognized potential weakness of DNA-based PCR, but the implications are purpose-dependent. With respect to raw milk analysis, in most cases the primary concern would be whether or not the milk was contaminated with salmonellae and not necessarily the viability of the bacteria. Any exposed milk would be treated as such regardless of potential bacterial viability.

It is also possible that the PCR technique is prone to false-positive reactions. Although in an assay such as the one described here, the primers and probes are very specific for one gene and the manufacturer has tested them against a wide range of bacterial genera, it is possible that milk contains other microbes that cross-react in this assay. Additional molecular tests based on a different gene might be used as confirmatory tests to back up the results obtained with the commercially available reagent kits used here.

The results of the present study indicate that it is important to evaluate the data (fluorescence plots and melting-point curves) for each sample prior to identifying that sample as *Salmonella* positive or *Salmonella* negative. The determination made by the RAPID is based on a "score" that is dependent on final fluorescence and baseline variability. In the present study, the calls were somewhat inconsistent for samples with low scores, and it is necessary to consult the plots before a final call is made. This is particularly true when the PCR response is very low. Should any response, regardless of its magnitude, be interpreted as positive? The answer probably depends on the individual sampling circumstance and the impact of a positive or negative result. In the present study, any response, regardless of magnitude, was considered positive.

When Bailey et al. (2) compared traditional manual

serotype identification and riboprint serotype identification, they determined that there was good agreement (90%) between the two systems, but only when *Salmonella* Montevideo was not included in the data set. *Salmonella* Montevideo was shown to have multiple ribotypes that were not discriminated by traditional serotyping. In the current study, *Salmonella* Montevideo was isolated from two samples that were determined to be *Salmonella* negative by real-time PCR. *Salmonella* Montevideo was also isolated from five other milk samples, but real-time PCR identified these samples as being *Salmonella* positive. On the basis of these observations, it appears that the primer-probe set used in this study may not have been universal enough to efficiently detect all Montevideo ribotypes. However, analysis of *Salmonella* Montevideo isolated from seven bulk milk samples showed that the commercially prepared reagents used in this study were able to detect all of these isolates, including isolates from the two samples for which *Salmonella* was not detected in the enrichment pellets when high concentrations of purified DNA were used. It is possible that in *Salmonella* Montevideo, small sequence differences within the gene targeted by the PCR primers and probes affect the efficiency of the real-time PCR, so that low levels of some Montevideo strains may not be detected.

On the basis of these results, real-time PCR appears to be a useful tool for detecting *Salmonella* in raw milk. The combination of enrichment and real-time PCR techniques used in this study resulted in the identification of more *Salmonella*-positive samples than traditional culture techniques did. Additionally, enrichment followed by real-time PCR can yield results in 24 h, as opposed to 48 to 72 h for traditional culture.

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